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Real-Time PCR for Detection and Identification of *Plasmodium* spp.

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Rapid and accurate detection of malaria parasites in blood is needed to institute proper therapy. We developed and used a real-time PCR assay to detect and distinguish four *Plasmodium* spp. that cause human disease by using a single amplification reaction and melting curve analysis. Consensus primers were used to amplify a species-specific region of the multicopy 18S rRNA gene, and SYBR Green was used for detection in a LightCycler instrument. Patient specimens infected at 0.01 to 0.02% parasitemia densities were detected, and analytical sensitivity was estimated to be 0.2 genome equivalent per reaction. Melting curve analysis based on nucleotide variations within the amplicons provided a basis for accurate differentiation of *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. For assay validation, 358 patient blood samples from the National University Hospital in Singapore and Evanston Northwestern Healthcare in Illinois were analyzed. Of 76 blinded patient samples with a microscopic diagnosis of *P. falciparum*, *P. vivax*, or *P. ovale* infection, 74 (97.4%) were detected by real-time PCR, including three specimens containing mixed *P. falciparum*-*P. vivax* infections. No *Plasmodium* DNA was amplified in any of the 82 specimens sent for malaria testing but that were microscopically negative for *Plasmodium* infection. In addition, 200 blood samples from patients whose blood was collected for reasons other than malaria testing were also determined to be negative by real-time PCR. Real-time PCR with melting curve analysis could be a rapid and objective supplement to the examination of Giemsa-stained blood smears and may replace microscopy following further validation.

Malaria remains a global concern. The World Health Organization estimates 300 to 500 million cases of malaria infections resulting in over one million deaths occurring globally each year. Although the vast majority of these cases are found in the 100 countries in the tropical regions of Africa, Asia, Central and South Americas where the disease is endemic, the mobile nature of today's society results in ca. 1,400 cases of imported malaria reported each year in the United States as a result of international travel, immigration, and military service (10). The Centers for Disease Control and Prevention recommends that malaria should be considered in the differential diagnosis of febrile patients who have traveled to a region where malaria is endemic and in any patients who experience fevers of unknown origin regardless of their travel history (10). Provision of these diagnostic services and maintenance of competency may pose a challenge to many laboratories.

Species differentiation of *Plasmodium* is essential for selecting the proper treatment. Especially important is differentiating *P. falciparum* from the others, since this species is responsible for ca. 95% of the deaths due to malaria (33). The current standard for diagnosis is the microscopic examination of Giemsa-stained thick and thin blood smears (12, 16, 21, 22, 28). This procedure is time-consuming to prepare, read, and interpret the slides. Previous studies have shown that even with experienced microscopists, misdiagnosis occurs, particularly in cases of mixed infection or low parasitemia (12, 28). Immunochromatographic assays based on antigen detection have been developed but are also relatively insensitive in cases of low parasitemia (22, 24, 30). In addition, antigenemia may persist weeks beyond the actual infection, leading to the false diagnosis of malaria parasitemia (6, 22). Molecular detection for *Plasmodium* diagnosis using PCR has resulted in increased sensitivity and species discrimination compared to either microscopic or immunochromatographic diagnosis of malaria (2, 4, 12, 14, 22, 23, 25, 27). However, most published PCR assays are gel based with (4, 5, 15) or without (2, 3, 9, 23, 25–27, 29, 31, 35, 36) subsequent probe hybridizations, resulting in a lengthy procedure not optimal for clinical use. The need for a more sensitive and time-efficient assay has led to the development of molecular assays involving real-time PCR (7, 8, 13, 18). Real-time PCR assays have the potential to detect low levels of parasitemia, identify mixed infections, and allow for precise differentiation of species via melting curve analysis. In the present study, we developed and validated a real-time PCR assay to detect and identify *Plasmodium* spp. in a single reaction by using a simple collection method consisting of blood spotted on treated filter paper.

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MATERIALS AND METHODS

Specimens. The study was approved by the Institutional Review Boards of Evanston Northwestern Healthcare (ENH) in Evanston, Ill., and the National University Hospital (NUH) in Singapore. Controls obtained from the American

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TABLE 1. Comparison of results between the original microscopic diagnoses and the microscopic review of a blinded subgroup containing discrepant cases

Original microscopic diagnosis	Final microscopic diagnosis (no. of specimens)[a]				
	P. falciparum	*P. malariae*	*P. ovale*	*P. vivax*	Negative
P. falciparum	34*	0	0	2	0
P. malariae	0	0	0	2	0
P. ovale	0	0	1	0	0
P. vivax	0	0	0	40*	0
Negative	0	0	0	0	82
Total	34*	0	1	44*	82

[a] *, Includes one specimen of a mixed *P. falciparum-P. vivax* infection.

Type Culture Collection (ATCC; Manassas, Va.) included plasmids containing a partial 18S rRNA gene sequence from each species (*P. falciparum* [MRA-177], *P. vivax* [MRA-178], *P. malariae* [MRA-179], and *P. ovale* [MRA-180]). In addition, 10 DNA samples from *P. falciparum*-infected human red blood cell cultures and monkey blood samples infected with *P. vivax* (five strains) or *P. malariae* (one strain) were also obtained from the ATCC. During the development of the assay, DNA was isolated from eight blood specimens known to contain either *P. falciparum* or *P. vivax* from patients at ENH or NUH. For validation, 200 blood samples from patients whose blood was collected for reasons other than malaria diagnosis and 158 blinded samples from patients with fever and a history of recent travel within a region where malaria infection is endemic that were obtained from ENH and NUH, including specimens from sources in Malaysia, Myanmar, and Thailand, were tested. Microscopic analysis was performed by experienced microscopists at each hospital. Thin and thick smears from specimens with an initial determination of *Plasmodium* infection were again blinded and analyzed by a panel of experienced microscopists from ENH and Northwestern Memorial Hospital in Chicago, Ill. On the basis of enlarged infected red blood cells, the occasional presence of Schüffner's dots, and the rare identification of characteristic schizonts, four cases initially reported as either *P. falciparum* or *P. malariae* were identified as *P. vivax* in the second analysis; the results of the other blinded specimens were in agreement for both the initial and second microscopic analyses. The final microscopic determinations of species are listed in Table 1 and were compared to the real-time PCR results.

Blood specimen preparation. Since the present study involved an international collaboration, the use of ISOCODE cards (Schleicher & Schuell, Keene, N.H.) containing spotted samples of fresh or EDTA-anticoagulated blood was the requested method for specimen collection. Approximately 40 μl of blood was applied to each circle of an ISOCODE card and allowed to dry completely. DNA from a single 6-mm hole punch (equivalent to one-fourth to one-half of an ISOCODE card circle) was extracted utilizing one wash with 500 μl of nuclease-free water, followed by a 30-min incubation at 95 to 100°C in 50 μl of nuclease-free water. Twenty blood samples that were clotted or thickened by prolonged storage before application to the ISOCODE cards were alternatively processed by using a Puregene (Gentra Systems, Minneapolis, Minn.) procedure for tissue samples according to the manufacturer's instructions. The specimens chosen for the Puregene procedure were distinguishable as the dried blood and/or clot remained on the surface of the card and did not penetrate the paper efficiently.

Real-time PCR. *Plasmodium* detection was performed by using real-time PCR in the LightCycler (Roche Molecular Systems, Indianapolis, Ind.). The 18S rRNA gene was chosen as the target since it contains both highly conserved and variable regions, and at least five copies of the gene are dispersed on separate chromosomes of the *Plasmodium* genome (11, 20). Consensus primers were designed after comparing several partial 18S rRNA gene sequences for each of four *Plasmodium* species (PL1473F18 [5′-TAA CgA ACg AgA TCT TAA-3′] and PL1679R18 [5′-gTT CCT CTA AgA AgC TTT-3′]; sequence numbering from GenBank accession number M19173). A BLAST analysis (1) of GenBank indicated that amplification with these two oligonucleotides would only occur when a *Plasmodium* spp. was available to act as a template (expected value of 0.31 for both oligonucleotides). Each 20-μl reaction mix contained 2 to 5 μl of sample DNA, 2 μl of 10× FastSTART DNA SYBR Green reagent (Roche), 6.5 mM $MgCl_2$ (final concentration), and 0.5 mM concentrations of each primer. The PCR conditions consisted of an initial denaturation at 95°C for 10 min, followed by amplification for 40 cycles of 10 s at 95°C, 5 s at 50°C, and 20 s at 72°C, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melt program consisting of 2 min at 95°C, 2 min at 68°C, and a stepwise temperature increase of 0.2°C/s until 90°C, with fluorescence acquisition at each temperature transition. The fluorescence data were analyzed by using F1/F2 settings, which improved the detection of *P. falciparum*, and a cutoff of 35 cycles was used to define *Plasmodium*-positive samples in the present study. This assay for *Plasmodium* species differentiation required ca. 1 h to complete, in addition to the sample preparation time. Melt curve analysis was used to determine the species-specific mean melting temperature (T_m) based on values determined from the respective plasmid controls.

RESULTS

Typical amplification and melt curves depicting T_m detection for the various species are shown in Fig. 1 to 5. Based on the published genome size for *P. falciparum* (22.8 Mbp) (11), Avogadro's number (6.022×10^{23} molecules per mole), and a standard nucleic acid molar conversion (1 μg of 1-kb DNA = 1.54 μmol), we calculated that 1 μg of *P. falciparum* DNA is equal to 4.067×10^7 genome copies or one genome copy in 24.6 fg of DNA. Control *Plasmodium* DNA samples obtained from in vitro culture in human reticulocytes and diluted to 0.2 genome copies (5 fg) per reaction were detected and correctly identified in the melting curve analysis; this is possible due to the presence of more than one copy of the 18S rRNA gene per genome. An excess amount (150 ng) of human DNA previously tested negative for *Plasmodium* was included as a negative control to estimate the possible background fluorescence in this SYBR Green assay.

Melting curve analysis permitted the clear identification of each *Plasmodium* species control, as shown in Fig. 2. The T_m values for the control plasmids were highly reproducible on eight repeated melt curve runs. Table 2 contains the average melting curve peak T_m for each *Plasmodium* spp. identified in the control plasmids and the other genomic DNA specimens used to develop the real-time PCR assay. Mixed reactions were created to mimic a dual infection with *P. falciparum* and *P. vivax*. Detection of each species was achieved at a ratio equivalent to 10 *P. falciparum*: one *P. vivax* (data not shown), a ratio that should approximate the number of *P. falciparum*-infected red blood cells that is generally higher in peripheral blood smears. DNA obtained from *P. vivax*- or *P. malariae*-infected monkey blood samples and 10 separate isolates of *P. falciparum* genomic DNA from the ATCC confirmed the reproducibility of the melting curve (Fig. 3). Four *P. falciparum* patient

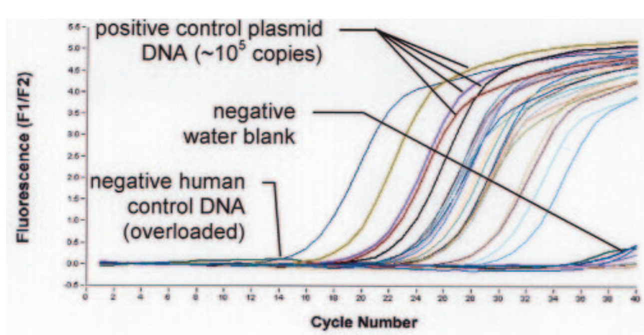

FIG. 1. Real-time amplification with SYBR Green fluorescence detection. The plasmid controls for four species, water blank, and negative human control DNA are indicated. The remaining curves are patient specimens with various parasitemia levels. The graph was generated by using LightCycler Software v. 3.

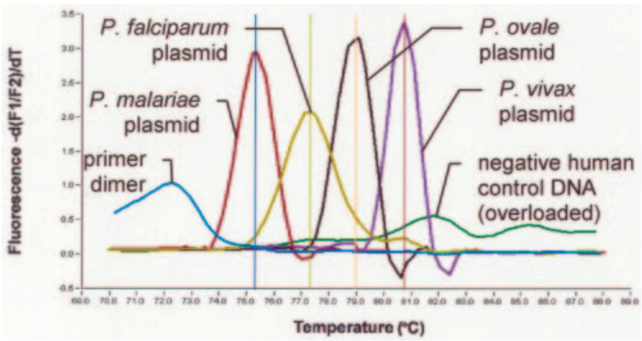

FIG. 2. Melting curve analysis, with the control plasmids, water blank, and negative human control DNA labeled. Vertical lines indicating the T_m values for each of the plasmid controls are continued in Fig. 3 through 5. The graph was generated by using LightCycler Software v. 3.

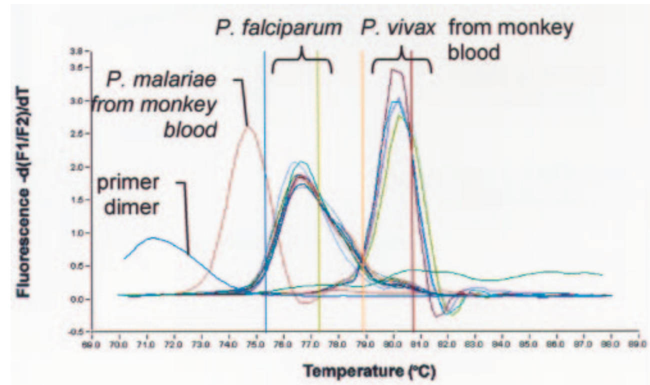

FIG. 3. Melting curve analysis: DNA isolated from blood by using the Puregene procedure from monkeys infected with either *P. malariae* or *P. vivax* (ATCC) and purified *P. falciparum* genomic DNA (ATCC). The graph was generated by using LightCycler Software v. 3.

samples and four *P. vivax* patient samples were also tested during assay development. We did observe a consistent slight shift to a higher T_m (up to 1°C) in the amplicons generated by the control plasmids compared to the other specimens tested (Table 2). The cause for this shift is currently unknown, although we assume sequence differences in the cloned fragments or variable salt concentrations in the DNA aliquots are responsible. Taking into consideration the T_m values for the control plasmids, the infected monkey blood, the infected human red blood cell cultures, and the eight patient samples, broad, nonoverlapping T_m ranges were selected to identify the *Plasmodium* species in the blinded patient specimens. The T_m values chosen were as follows: *P. malariae*, 73.5 to 75.5°C; *P. falciparum*, 75.5 to 77.5°C; *P. ovale*, 77.5 to 79.0°C; and *P. vivax*, 79.0 to 81.0°C. DNA prepared by using the Puregene procedure had T_m values universally lower by ca. 1°C than DNA prepared from identical specimens using the standard ISOCODE extraction protocol but was still at the lowest end of the broad ranges described above. These lower T_m values are most likely due to the salt content in the Puregene DNA hydration buffer compared to the nuclease-free water of the ISOCODE card protocol.

Parasites in 74 of 76 (97.4%) blinded patient samples with a positive microscopic diagnosis of malaria were detected by real-time PCR (Fig. 4), including three specimens containing mixed *P. falciparum*-*P. vivax* infections. Two samples that were diagnosed by microscopy as only *P. falciparum* infections were identified as mixed *P. falciparum*-*P. vivax* infections by real-time PCR (Fig. 5). A single patient infected with *P. ovale* was identified by both microscopic analysis and real-time PCR (microscopic identification of *P. ovale* confirmed by the Illinois Department of Public Health). Two specimens microscopically diagnosed as containing *P. falciparum* with parasitemia levels of ca. 0.01% (data not shown) did not produce an amplification curve within the 35 cycle cutoff limit used for the present study, even when the amount of template DNA used in each reaction was increased from 2 to 5 μl. Thus, the detection sensitivity for patient samples was estimated to be in the range of 0.01 to 0.02% parasitemia. Using the normal range of 4.7 to 6.1 million red blood cells per ml of blood, 0.01 to 0.02% parasitemia would be equivalent to 470 to 1,220 parasites per ml, or approximately 1 parasite per μl. This sensitivity corresponds favorably to other published methods, specifically real-time PCR assays.

Four samples were determined by initial microscopy to be two *P. malariae*- and two *P. falciparum*-infected specimens; all four were identified as *P. vivax* by the real-time PCR assay, which is in agreement with the final consensus identification for these four samples. Eighty-two specimens from patients

TABLE 2. Average melting curve peak T_m values for each *Plasmodium* sp.

Species	Avg T_m peak ± SD (°C)[a] in:	
	Plasmid clones*	Patient specimens
P. falciparum	77.0 ± 0.2	75.9 ± 0.4
P. malariae	75.0 ± 0.4	74.8 ± 0.4†
P. ovale	78.6 ± 0.3	78.5 ± 0.1*
P. vivax	80.4 ± 0.3	79.6 ± 0.5

[a] *, single DNA specimens averaged over several runs on separate days; †, no patients infected with *P. malariae* were identified by using the real-time PCR assay, so this value represents a single *P. malariae*-infected monkey blood specimen obtained from the ATCC and averaged over several runs on separate days.

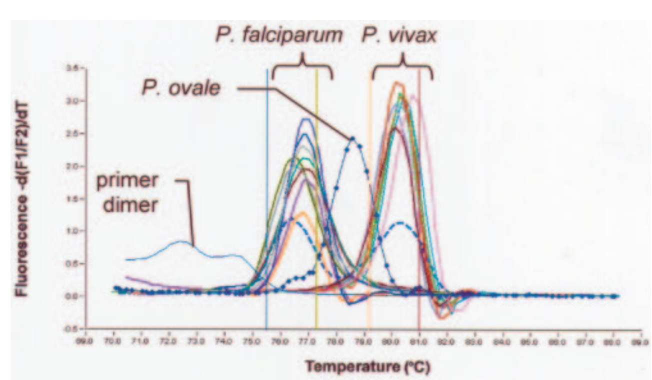

FIG. 4. Melting curve analysis: patient samples infected with *P. falciparum*, *P. ovale*, or *P. vivax* prepared by using the ISOCODE card procedure for DNA extraction. Patient sample infected with both *P. falciparum* and *P. vivax* is highlighted with a dashed line, and the patient specimen with *P. ovale* is highlighted with solid circle points. The graph was generated by using LightCycler Software v. 3.

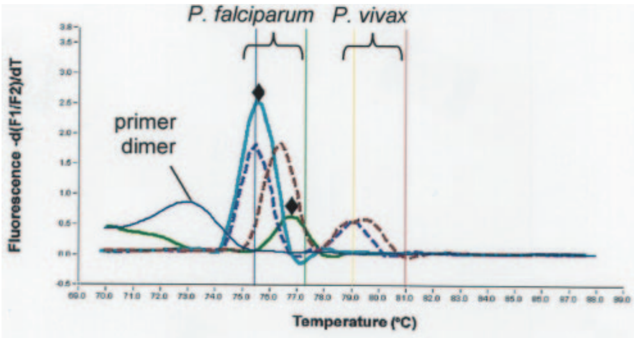

FIG. 5. Melting curve analysis: discordant patient samples. Dual *P. falciparum* and *P. vivax* infections not microscopically identified are highlighted with dashed lines. The blue dashed line represents a specimen prepared by using the Puregene method, and the red dashed line is one prepared by using the standard ISOCODE method, exemplifying the slightly lower T_m peaks resulting from the former method. The black diamonds indicate microscopically diagnosed *P. falciparum* specimens that amplified beyond the 35 cycle cutoff limit used in the present study. The graph was generated by using LightCycler Software v. 3.

with clinical findings suggesting malaria but microscopically negative for *Plasmodium* and subsequently determined not to be infected were PCR negative. An additional 200 blood samples from patients collected for diagnoses unrelated to malaria (data not shown) were also determined to be negative by real-time PCR. Using results from the second microscopic analysis as the gold standard, the sensitivity, specificity, positive and negative predictive values, and accuracy of the real-time PCR assay for each detected *Plasmodium* spp. are summarized in Table 3.

DISCUSSION

Although there are many published studies showing the improved sensitivity and specificity of PCR-based assays over microscopic or immunochromatographic diagnosis of malaria (2, 4, 12, 14, 22, 23, 25, 27), only a handful take advantage of the even more sensitive and time-efficient real-time PCR technologies. In several studies in which real-time PCR was utilized, either a single-species was identified (13) or no distinction between the four human parasites was made (8, 18). This distinction is critical in the clinical management of patients, since the course of treatment varies depending on which species is the cause of the infection. Only in the study by de Monbrison et al. were the four human parasites identified by real-time PCR (7). This assay included five separate primer pairs amplified simultaneously in a LightCycler instrument, utilizing melting curve analysis to distinguish the *Plasmodium* spp. In the de Monbrison study, twenty-nine single infections and four dual infections were identified by real-time PCR. Twelve specimens were negative by both microscopy and real-time PCR.

The single reaction real-time PCR assay described in our study used only a single pair of primers to detect and identify *Plasmodium* spp. in 3 h, including standard DNA sample preparation, amplification, and detection, with sensitivities equivalent to microscopy. An additional 1 to 2 h was needed when the more extensive Puregene DNA preparation was required. As with several other studies, we chose to target a species-specific region of the *Plasmodium* 18S (small subunit) rRNA gene in our real-time PCR assay. By targeting a gene with multiple copies dispersed throughout the *Plasmodium* genome (11), the sensitivity of this assay is greater than if a single-copy gene was chosen. We also chose to target the partial 18S rRNA gene sequence included in the cloned plasmids available from the ATCC to permit easy access to individual species-specific controls. Although several fluorescence resonance energy transfer hybridization probes and different primer sets were tested in the development of this assay (data not shown), the described SYBR Green assay provided the best discrimination among the different species. The sequences bracketed by the primers are sufficiently divergent in the four species to provide at least a 1°C difference between the average T_m values to distinguish between the species during the melting curve analysis. Single base differences theoretically may also exist between strains within a species from different geographic regions, but our results indicate that the variances among individual strains or patient specimens prepared by the same DNA purification procedure are minor in comparison to the differences among species. Additional specimens from other regions around the world would be required to confirm these assumptions.

We did not address the analysis of blood samples collected by various methods in the present study. To obtain a diverse group of patient specimens, blinded samples from ENH and NUH were collected on ISOCODE cards and processed in the Molecular Diagnostics laboratory at ENH. We overcame potential problems posed by blood samples that were clotted or thickened due to long-term storage at 4°C before application to cards, thus failing to percolate into the filter paper adequately, by processing the "clumps" using the Puregene procedure for tissue samples. Samples with significant red blood cell lysis as a result of freezing were found to be unsuitable for analysis (data not shown). In the present study, DNA was isolated from ca. 10 μl of blood from each ISOCODE card specimen, and 1/50 to 1/20 of this DNA was used in each real-time PCR.

We analyzed 76 blood specimens from patients with clinical findings suggesting malaria for which a microscopic determination of *Plasmodium* infection had been identified. We, like others, had some difficulty in arriving at a consensus for the microscopic diagnosis. Our final microscopic diagnosis used for comparison was based on review by a panel of experienced microscopists and is reported in Table 1. Real-time PCR anal-

TABLE 3. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the real-time PCR assay relative to microscopic analysis using the results from the morphologic assessment of a panel of experienced microscopists

Parameter[a]	%[b]			
	P. falciparum	*P. malariae*	*P. ovale*	*P. vivax*
Sensitivity	94.1	Unknown	100.0	100.0
Specificity	100.0	Unknown	100.0	99.1
PPV	100.0	Unknown	100.0	95.5
NPV	98.4	Unknown	100.0	98.3
Accuracy	98.7	Unknown	100.0	98.1

[a] PPV, positive predictive value; NPV, negative predictive value.
[b] Only one patient here was infected with *P. ovale*, as determined by microscopic analysis; no patients were infected with *P. malariae*.

ysis identified *P. falciparum*, *P. vivax*, or *P. ovale* in 74 (97.4%) of these specimens, including 3 that contained a dual infection with *P. falciparum* and *P. vivax*, for which only 1 had been microscopically identified as such. Since it is often difficult to diagnose mixed infections by microscopy, the distinct dual peaks observed in Fig. 5 indicate that the real-time PCR assay may be more accurate than the gold standard of microscopy in these two cases. To date, only a single patient with a *P. ovale* infection had been identified by microscopy and real-time PCR. Of the two specimens microscopically diagnosed as containing *P. falciparum* that did not produce a positive result for any *Plasmodium* infection in the real-time PCR assay, the amount of parasitemia in these samples was ca. 0.01%, at the lower threshold of sensitivity for this assay. In both cases, the blood had been stored for several days before aliquots were spotted onto the ISOCODE cards. It is also possible that the sensitivity of the assay was decreased by the delay in specimen preparation, in spite of using the Puregene procedure.

Of the 74 infected specimens detected by both microscopy and real-time PCR, 4 had discrepant species identification based on the initial microscopic analysis. Two specimens with *P. malariae* and two with *P. falciparum*, as originally determined by microscopic analysis, were all identified as *P. vivax* positive by the real-time PCR assay. GenBank sequence comparisons and the results of preliminary testing using ATCC controls would suggest that misidentification of either *P. falciparum* or *P. malariae* as *P. vivax* is unlikely using this sequence-based assay and may indicate an inaccurate microscopic diagnosis. The four discrepant specimens were included in a blinded group reviewed by a panel of experienced microscopists. The results of this second review were in agreement with the real-time PCR results, indicating the real-time PCR assay may provide accurate identification of *Plasmodium* spp. when the microscopic analysis is uncertain.

Recently, *P. knowlesi*, a species that is morphologically similar to *P. malariae* has been identified as a human pathogen in patients from Malaysia (32). Sequence analysis indicates that this species should also be amplified by using the conserved primers described in this assay. Whether the differences in sequence composition between the primers are sufficient to result in a T_m closer to that of *P. vivax* than to *P. malariae* needs to be determined by testing confirmed *P. knowlesi* specimens.

A drawback common to all DNA-based malaria assays is the real possibility of detecting persistent DNA in the bloodstream after the infection has subsided. Several studies reported the posttreatment detection of *Plasmodium* DNA up to 4 days after microscopic detection (4, 25), and another purports that detection at 5 to 8 days after initiation of treatment indicates therapeutic failure, possibly due to parasite resistance (17). However, this persistence of *Plasmodium* DNA is still less than the persistent antigenemia reported with immunochromatographic assays (6, 22). Also, DNA-based molecular assays have demonstrated a possible chronic detection in regions of the world where malaria is endemic, which may be due to very low parasitemias in immune individuals (3, 19, 34).

In the present study, we developed and validated a real-time PCR assay that can accurately detect and identify *Plasmodium* spp. in a single reaction by using a simple method for sample collection consisting of blood spotted on treated filter paper (Table 3). The entire process, including specimen preparation using the standard ISOCODE card procedure, PCR amplification, and melting curve identification of *Plasmodium* spp., was completed in less than 3 h. This real-time PCR assay for the detection and identification of *Plasmodium* spp. can be used to confirm microscopic findings and in many settings can be used for the primary identification of an infected patient without the need for multiple blood specimens or even microscopic analysis.

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